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Inhibition of Cell Division by the Human Cytomegalovirus IE86 Protein: Role of the p53 Pathway or Cyclin-Dependent Kinase 1/Cyclin B1

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The human cytomegalovirus (HCMV) IE86 protein induces the human fibroblast cell cycle from G_0/G_1 to G_1/S, where cell cycle progression stops. Cells with a wild-type, mutated, or null p53 or cells with null p21 protein were transduced with replication-deficient adenoviruses expressing HCMV IE86 protein or cellular p53 or p21. Even though S-phase genes were activated in a p53 wild-type cell, IE86 protein also induced phospho-Ser_{15} p53 and p21 independent of p14ARF but dependent on ATM kinase. These cells did not enter the S phase. In human p53 mutant, p53 null, or p21 null cells, IE86 protein did not up-regulate p21, cellular DNA synthesis was not inhibited, but cell division was inhibited. Cells accumulated in the G_2/M phase, and there was increased cyclin-dependent kinase 1/cyclin B1 activity. Although the HCMV IE86 protein increases cellular E2F activity, it also blocks cell division in both $p53^{+/+}$ and $p53^{-/-}$ cells.

The DNA tumor viruses such as adenovirus, simian virus 40, and the papillomaviruses have the ability to transform cells (38). These viruses inactivate the Rb family of proteins to induce E2F-responsive gene expression (11, 23, 49). For regulation of cellular proliferation, the Rb pathway is functionally linked to the p53 pathway (50). Deregulated E2F activity induces the p53 pathway to inhibit cell cycle progression or to induce apoptosis. By doing so, the growth and development of cancerous cells are prevented. The DNA tumor viruses interfere with the activity of p53 and escape a cellular checkpoint system induced by deregulated E2F activity (11, 23, 49).

p53 transactivates a set of genes that promote antiproliferative responses, including cell cycle arrest either at the G_1/S transition or at G_2 before mitosis (1, 5, 13, 43). The cyclin-dependent kinase (cdk) inhibitor p21 (Cip1) is one of the p53-responsive genes that play an important role in regulation of cellular proliferation (21, 51, 52, 60). Besides cdk inhibition, p21 also binds to proliferating cell nuclear antigen and inhibits DNA polymerase delta (14, 59, 60).

Human cytomegalovirus (HCMV), a member of the *Betaherpesvirus* family, has various effects on cellular physiology, including the cell cycle (7, 26, 56). HCMV productively infects terminally differentiated cells that are in the G_0/G_1 phase of the cell cycle and low in nucleotide triphosphate precursors for DNA synthesis. Although HCMV induces human foreskin fibroblast (HFF) cells to express genes required for S phase entry, cellular DNA synthesis does not occur in HFF cells (4, 7, 12, 24, 26, 29, 54, 56).

The viral immediate-early (IE) protein of approximately 86 kDa (IE86) is encoded by the *IE2* gene (UL122) of HCMV and is a strong transactivator of viral and cellular promoters. The IE86 protein interacts with the cellular basal transcription factors to activate transcription (9, 25, 30, 31). The IE86 protein also interacts with Rb, inhibits Rb-mediated repression of transcription (16, 20), and consequently up-regulates the expression of E2F-responsive genes (7, 26, 56). Despite up-regulated E2F-responsive gene expression, the IE86 protein also inhibits cellular DNA synthesis in HFF cells (40). The IE86 protein induces the level of p53 and interacts with p53 (3, 8, 55, 58).

To better understand how the IE86 protein of HCMV stimulates the S phase genes but either blocks cellular DNA synthesis or cell division, we determined the effect of the HCMV IE86 protein on $p53^{+/+}$ and $p53^{-/-}$ cells. Here we present data showing that the viral IE86 protein induces the level of phospho-Ser_{15} p53 and p21 in $p53^{+/+}$ cells, indicating that the p53 pathway is not impaired by the IE86 protein. The IE86 protein inhibits cellular DNA synthesis in $p53^{+/+}$ cells but not in $p53^{-/-}$ cells. $p53^{-/-}$ cells accumulate in the G_2/M phase, and there are aberrant levels of cdk1/cyclin B1 activity.

MATERIALS AND METHODS

Cell culture, virus, and adenovirus vectors. Primary HFFs were grown in Eagle's minimal essential medium (Mediatech, Herndon, Va.) supplemented with either 10% (high) or 0.05% (low) newborn calf serum (Sigma, St. Louis, Mo.) and penicillin (100 U/ml) and streptomycin (100 μg/ml). U373MG, a human glioblastoma-astrocytoma cell line, and U2OS (a gift from Dawn Quelle, University of Iowa), a human osteosarcoma cell line, were grown in Dulbecco's minimal essential medium (Mediatech) supplemented with either 10% (high) or 0.1% (low) fetal calf serum (JRH Biosciences, Lenexa, Kans.) and penicillin and streptomycin as described above. Saos-2, a human osteosarcoma cell line, was obtained from the American Type Culture Collection (Manassas, Va.) and grown in McCoy's 5A medium (Invitrogen, Grand Island, N.Y.) supplemented with either 15% (high) or 0.1% (low) fetal calf serum (JRH Biosciences) and penicillin and streptomycin as described above. Human dermal fibroblast cells from ataxia-telangiectasia (AT) patients were obtained from the Coriell Institute for Medical Research (Camden, N.J.) and grown in Eagle's minimal essential medium (Mediatech) supplemented with either 15% (high) or 0.05% (low) noninactivated fetal calf serum (Invitrogen) and penicillin and streptomycin as described above. HCT116, a human colon cancer cell line, and isogenic $p53^{-/-}$ or $p21^{-/-}$ mutant cells (a gift from Bert Vogelstein, Johns Hopkins University) were grown in McCoy's 5A medium (Invitrogen) supplemented with either 10% (high) or 0% (low) fetal calf serum (JRH Biosciences) and penicillin and strep-

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tomycin as described above. For serum starvation, cells were washed twice with serum-free medium and grown in a low-serum medium for 48 h.

Replication-defective E1a$^-$, E1b$^-$, and E3$^-$ adenovirus vector Ad-p53 and Ad-p21 were obtained from the University of Iowa Vector Core and the University of Michigan Vector Core, respectively. Replication-defective E1a$^-$, E1b$^-$, and E3$^-$ adenovirus vectors expressing either the Tet-off transactivator (Ad-Trans), the green fluorescent protein (Ad-GFP), or the HCMV IE86 protein (Ad-IE86) were described previously (36). Adenovirus vectors were grown in 293 cells and purified as described previously (36). The titers of the various recombinant adenovirus vectors were determined by plaque assay on 293 cells. For transduction experiments, 10 PFU of recombinant adenovirus vectors per cell in a serum-free medium containing 3 μl of Lipofectamine reagent (Life Technologies, Gaithersburg, Md.) per ml was used. After 1 h at 37°C, the inoculum was removed and the cells were maintained in media as described above.

Western blot analysis. Cells were collected, fractionated, and transferred to nitrocellulose membranes as described previously (36). Mouse monoclonal antibody to p53 (Oncogene, San Diego, Calif.), phospho-Ser$_{15}$ p53 (Cell Signaling Technology, Beverly, Mass.), cyclin B1 (BD Biosciences, San Diego, Calif.), or β-tubulin (Developmental Studies Hybridoma Bank, University of Iowa) or rabbit polyclonal antibody to p21 (Santa Cruz Biotechnology, Santa Cruz, Calif.) or cdk1/cdc2 (Oncogene) was used. Enhanced chemiluminescence detection reagents (Pierce, Rockford, Ill.) and secondary peroxidase-labeled anti-mouse or anti-rabbit immunoglobulin G antibody (Amersham Biosciences, Piscataway, N.J.) were used in accordance with the manufacturer's directions.

BrdU labeling and immunofluorescence assay. Serum-starved cells were transduced at 10 PFU/cell with either Ad-IE86, Ad-Trans, Ad-p53, or Ad-p21 plus Ad-Trans (10 PFU/cell), plated at low density on glass coverslips, and grown in high-serum medium with 10 μM bromodeoxyuridine (BrdU; Sigma) to allow reentry into the cell cycle. After 18 h of incubation at 37°C, cells were fixed as described previously (27) and stained with either rabbit anti-HCMV IE86 (6655; a gift from Jay Nelson, Oregon Health Science University), anti-human p53 (Santa Cruz Biotechnology), or anti-human p21 (Santa Cruz Biotechnology) antibody, followed by fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin (Santa Cruz Biotechnology). The cells were stained with a mouse monoclonal antibody to BrdU (Amersham Biosciences), and BrdU incorporation was detected with a Texas red-conjugated anti-mouse immunoglobulin (Santa Cruz Biotechnology). After staining, cells were visualized with an Olympus BX-51 light microscope (Central Microscopy Research Facility, University of Iowa).

Flow cytometry and colony-forming assay. Flow cytometric and colony-forming assays were carried out as described previously (36).

Immunoprecipitation and in vitro kinase assay. Three million cells were lysed by freezing and thawing with NP-40 lysis buffer (50 mM Tris-HCl [pH 8.0], 125 mM NaCl, 1 mM EDTA, 0.5% NP-40) containing protease and phosphatase inhibitors as described previously (33). Cell lysates were precleared with protein A agarose beads (Upstate, Lake Placid, N.Y.) and incubated at 4°C for 2 h with anti-cdk1/cdc2 antibody (Oncogene) plus protein A agarose beads. Immunoprecipitates were washed three times with NP-40 lysis buffer and twice with cdk buffer (50 mM HEPES [pH 8.0] containing 10 mM $MgCl_2$). Kinase assays were at 30°C for 30 min in cdk buffer containing 2 μg of purified histone H1 (Roche, Indianapolis, Ind.), 3 μM ATP, and 5 μCi of [γ-^{32}P]ATP. Reactions were stopped by addition of an equal volume of 2× sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer (200 mM Tris-HCl [pH 7.8] containing 8% sodium dodecyl sulfate, 0.02% bromophenol blue, and 20% β-mercaptoethanol). Samples were fractionated and transferred to nitrocellulose membranes. Western blot analysis was performed as described previously (36).

RESULTS

Effect of the IE86 protein on the levels of phospho-Ser$_{15}$ p53 and p21 in various cell types. Previous reports have shown that HCMV infection of permissive cells increases the level of p53 and phospho-Ser$_{15}$ p53 (17, 24, 35, 48, 55), but the levels of p21 decreased (10, 40). The IE86 protein of HCMV also increases the level of p53 and interacts with p53 (3, 8, 55, 58). We determined the effect of the IE86 protein on cell cycle regulation in p53$^{+/+}$ and p53$^{-/-}$ cells. HFF cells, in which the p53 pathway is intact, were transduced with either Ad-GFP, Ad-p53, or Ad-p53 plus Ad-IE86 and tested for the expression of p53, phospho-Ser$_{15}$ p53, and p21 by Western blotting. We used Ad-IE86 at 10 PFU/cell to express the viral IE86 protein at a level similar to that achieved by infection of HFF cells with HCMV at a multiplicity of infection of approximately 5 PFU/cell (data not shown). We used Ad-p53 because HFF cells normally have a low relative level of p53. The level of phospho-Ser$_{15}$ p53, as well as endogenous p21, was induced in HFF cells transduced with Ad-IE86 plus Ad-p53 compared to Ad-GFP plus Ad-p53 (Fig. 1A). The IE86 protein increased the level of endogenous p21 fourfold compared to that achieved with p53 alone (Fig. 1A). In the absence of exogenous p53, the IE86 protein induced a lower level of endogenous p21 in HFF cells (data not shown).

In cells under stress, p53 is stabilized to induce antiproliferative effects including cell cycle arrest, cellular senescence, and apoptosis (5, 43, 45). Cellular p14^{ARF} (p19^{ARF} in the mouse) and/or ATM kinase are known to induce p53 stabilization (1, 5, 44). Mdm2 (Hdm2 in humans) is a negative regulator that binds and targets p53 for degradation (1, 5, 43–45). p14^{ARF} binds to Hdm2 to antagonize its function and to free p53 (50). ATM kinase induces the phosphorylation of p53 at serine$_{15}$ to stabilize and activate p53 (1). Both p14^{ARF} and ATM are E2F-responsive genes, and deregulated E2F activity induces p14^{ARF} and/or ATM to activate the p53 pathway (2, 28, 32, 46). Since the IE86 protein induces E2F activity, we determined whether p14^{ARF} or ATM kinase is required for induction of the p53 pathway by the viral IE86 protein. We used U2OS cells, human osteosarcoma cells that are p14^{ARF} null, and AT cells, human dermal fibroblast cells from AT patients that lack a functional ATM kinase. These cells were transduced with either Ad-IE86 or Ad-GFP and tested for the expression of p53, phospho-Ser$_{15}$ p53, or p21 by Western blotting. In the U2OS cells, the IE86 protein induced p53, phospho-Ser$_{15}$ p53, and p21 5-, 3-, and 10-fold, respectively (Fig. 1B). In contrast, there was little to no induction of p53, phospho-Ser$_{15}$ p53, and p21 in the AT cells (Fig. 1B). The basal level of p53 was induced at a low level by the IE86 protein in the AT cells, and p14^{ARF} may play a minor role to increase the level of p53 by the IE86 protein in the absence of ATM kinase. Nevertheless, these data indicate that the ATM kinase plays a major role in the induction of the p53 pathway by the IE86 protein.

Since p21 can be induced by a p53-independent mechanism (19, 22), we determined whether the IE86 protein induces endogenous p21 in the absence of functional p53. U373MG cells, human glioblastoma-astrocytoma cells that are Rb and p53 mutant (18), or Saos-2 cells, human osteosarcoma cell that are Rb mutant and p53 null (53), were transduced with either Ad-IE86, Ad-GFP, or Ad-p53 and tested for expression of p21 by Western blotting. IE86 protein did not induce p21 in the cells lacking functional p53. However, expression of exogenous p53 by Ad-p53 induced expression p21 in these cells (Fig. 1C). These data indicate that IE86 protein induces p21 through the p53 pathway. The IE86 protein induces the p53 pathway independent of p14^{ARF} but dependent on ATM kinase.

Effect of the IE86 protein on cellular DNA synthesis in p53$^{+/+}$ or p53$^{-/-}$ cells. A previous report has shown that the IE86 protein inhibits cell cycle progression at the G_1 phase in HFF cells (40). In contrast, the IE86 protein induces the S phase in U373MG cells that are p53 mutant (36). The differ-

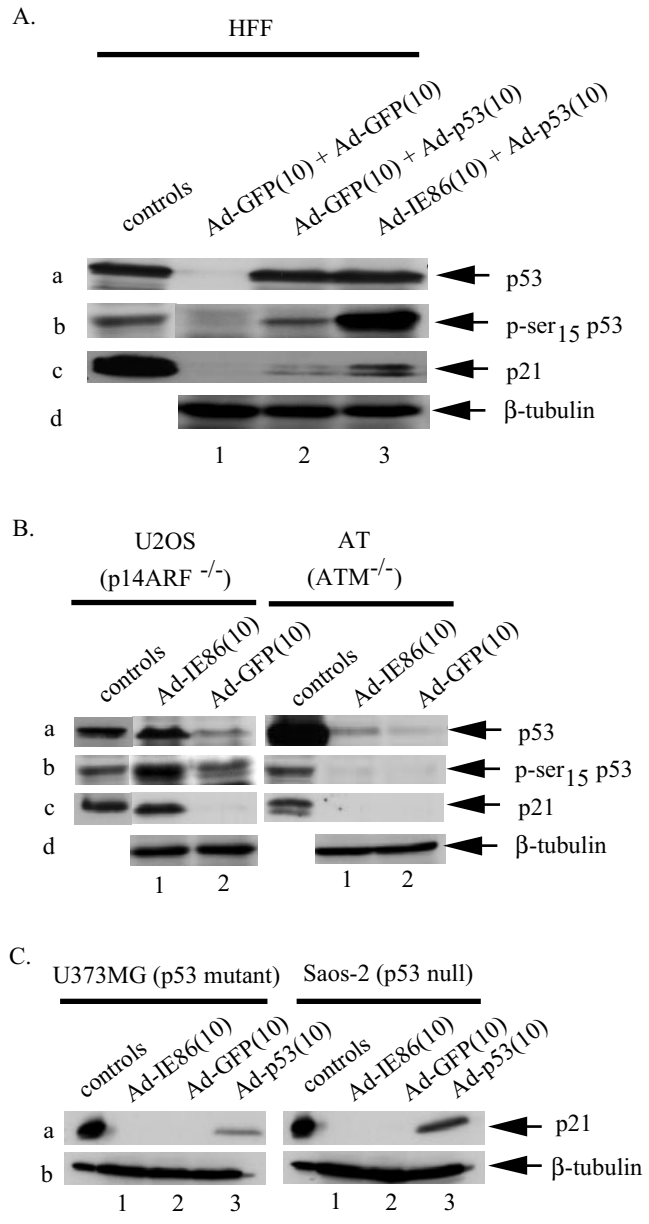


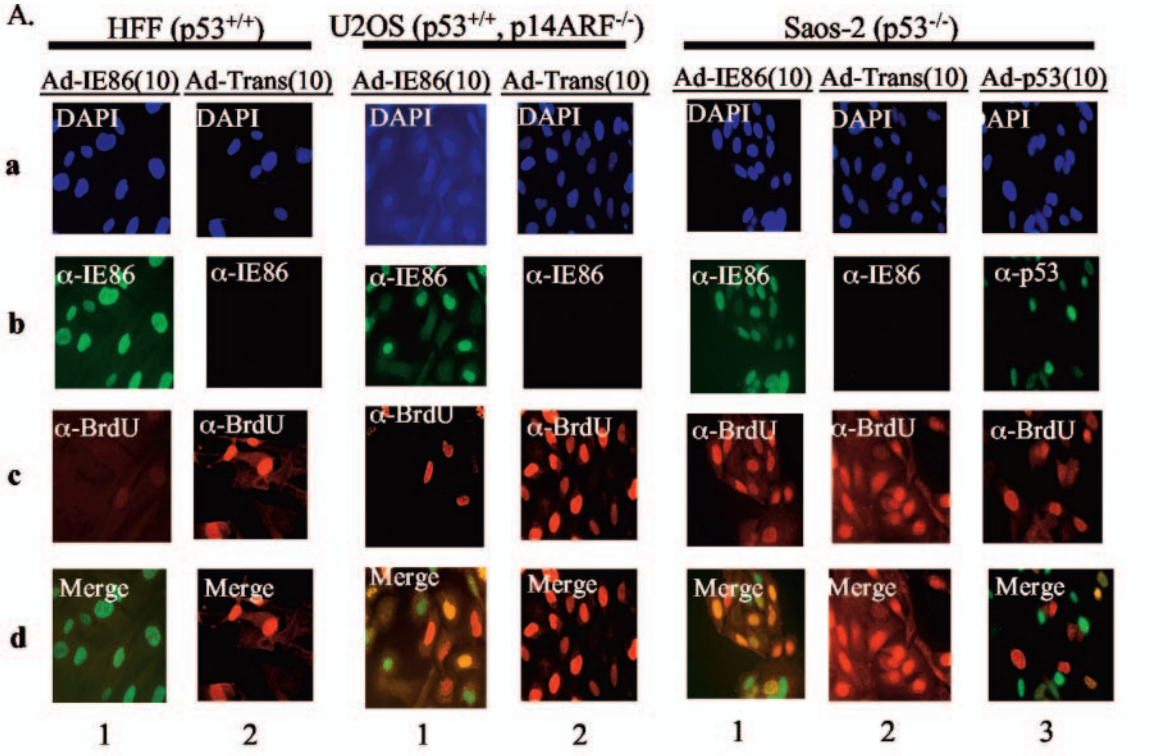
FIG. 1. Effect of IE86 protein on levels of phospho-Ser_{15} p53 and p21 in various cell types. Cells were transduced at 10 PFU/cell with either Ad-GFP, Ad-p53, or Ad-p53 plus Ad-IE86 in the presence of Ad-Trans (10 PFU/cell). Western blot analysis was done with antibody to p53, phospho-Ser_{15} p53, p21, or β-tubulin as described in Materials and Methods. The positive controls for p53 and p21 are HFF cells transduced at 10 PFU/cell with either Ad-p53 or Ad-p21, respectively. For a positive phospho-Ser_{15} p53 control, HFF cells were treated with 20 mM hydroxyurea for 48 h. (A) HFF cells. (B) $p14^{ARF-/-}$ U2OS or $ATM^{-/-}$ AT cells. (C) p53 mutant U373MG or p53 null Saos-2 cells.

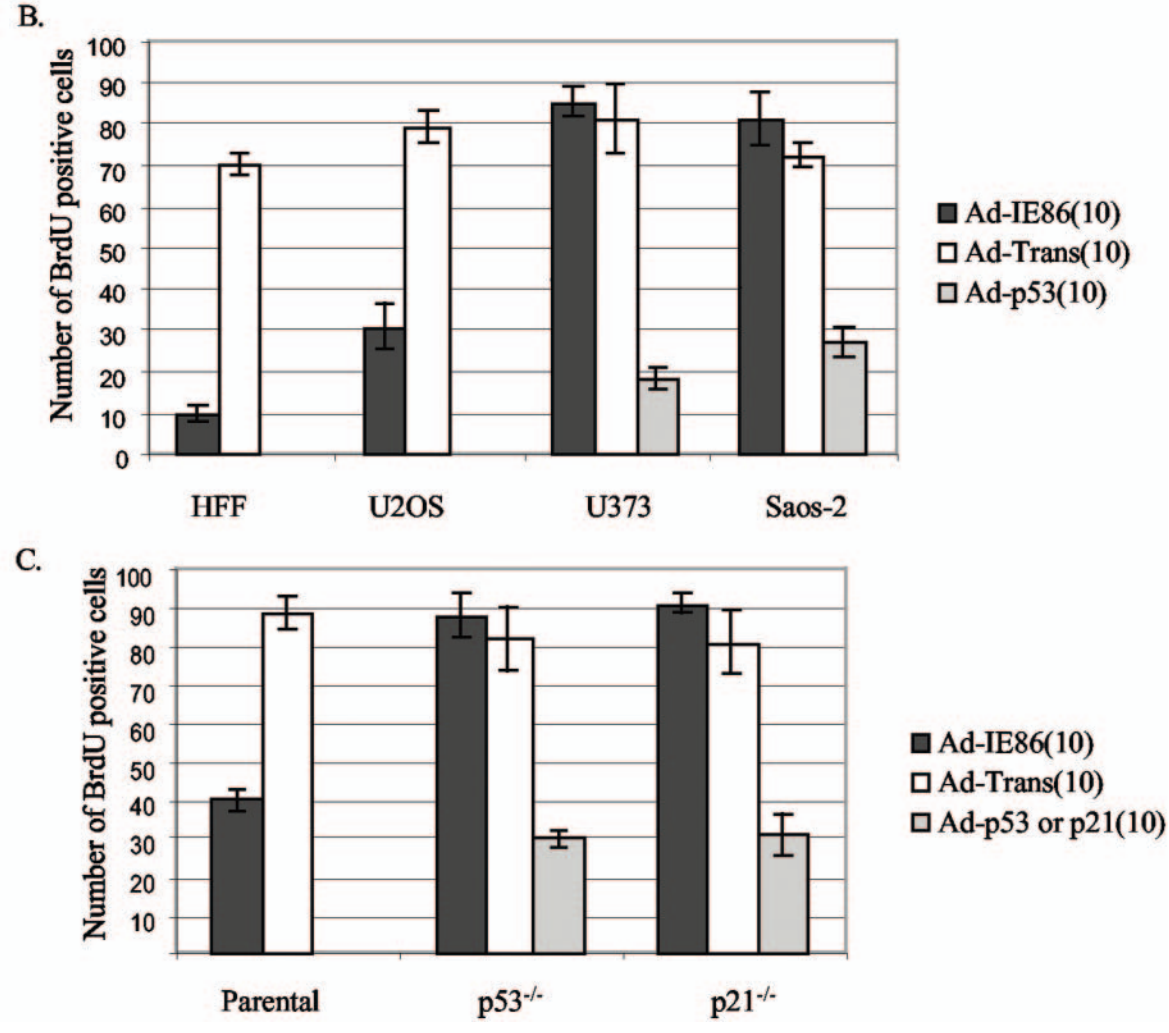
ences in these results could be due to the p53 status in the different cell types. To determine the effect of IE86 protein on cell cycle progression, various cell types with or without functional p53 were transduced with Ad-IE86 and cellular DNA synthesis in these cells was determined. HFF, U2OS, U373MG, or Saos-2 cells were synchronized at the G_1 phase by serum starvation; transduced with either Ad-IE86, Ad-Trans, or Ad-p53; serum induced; and incubated with BrdU. After 24 h, incorporation of BrdU was measured by immunofluorescence assay and BrdU-positive cells were counted. Cellular DNA synthesis in HFF or U2OS cells, which have functional p53, was significantly reduced by the IE86 protein (Fig. 2A). In contrast, the IE86 protein did not inhibit cellular DNA synthesis in U373MG cells, which are p53 mutant (data not shown), or Saos-2 cells, which are p53 null (Fig. 2A). Inhibition of cellular DNA synthesis in these cells was restored by expression of p53 with Ad-p53 (Fig. 2A and data not shown). Figure 2B summarizes the number of BrdU-positive cells and the effect of IE86 protein or p53 in HFF, U2OS, U373MG, and Saos-2 cells.

Since transformed cells can have mutations in more than one cellular protein regulating cellular proliferation, we also tested HCT116 cells and isogenic mutant cells that lack either p53 or p21 through specific knockout of these genes by homologous recombination (6). HCT116 cells are human colon cancer cells that contain a functional p53 pathway. Cellular DNA synthesis in parental HCT116 cells, but not in isogenic $p53^{-/-}$ or $p21^{-/-}$ mutant cells, was inhibited by the IE86 protein (Fig. 2C). Introduction of exogenous p53 or p21 with Ad-p53 or Ad-p21 into $p53^{-/-}$ or $p21^{-/-}$ cells, respectively, inhibited cellular DNA synthesis to approximately the same level as exogenous IE86 protein introduced with Ad-IE86 into the parental cells (Fig. 2C). Taken together, these data indicate that the viral IE86 protein inhibits cellular DNA synthesis via a functional cellular p53 to arrest cell cycle progression.

Effect of IE86 protein on $p53^{+/+}$ or $p53^{-/-}$ cell division. Since cellular DNA synthesis is not inhibited in cells that lack functional p53, we determined whether IE86 protein can inhibit cell division. Twenty thousand cells transduced with either Ad-GFP or Ad-IE86 were grown for 6 days, and a colony-forming assay was performed to test cell division as described in Materials and Methods. Regardless of the p53 or p21 status of the cell, the IE86 protein inhibited cellular division in greater than 95% of the cells, which is similar to the adenovirus vector transduction efficiency at 10 PFU/cell (Fig. 3). These data indicate that, in the absence of the p53 pathway, the viral IE86 protein uses another mechanism to inhibit cell division.

Effect of IE86 protein on cell cycle progression in $p53^{+/+}$ or $p53^{-/-}$ cells. We had previously reported that the IE86 protein stops the U373MG cell cycle in the S phase (36). Since the mutant p53 may have unknown effects on cell cycle progression, we determined the nature of the cell cycle block by the IE86 protein in the absence of the p53 pathway with Saos-2 cells, which are p53 null. Serum-starved HFF or p53 null Saos-2 cells were transduced with either Ad-IE86 or Ad-Trans control and incubated with high-serum medium. After 48 h, the cells were stained for DNA content with propidium iodide and cell cycle analysis was performed by fluorescence-activated cell sorter scanning as described in Materials and Methods. In HFF, an increase in the G_1 phase of the cell cycle of the Ad-IE86-transduced cells relative to that of the Ad-Trans-transduced cells (approximately 69 versus 56%, respectively) was detected as reported previously (40). In contrast, p53 null Saos-2 cells transduced with Ad-IE86 had a significant increase in the G_2/M phase component compared to cells transduced with Ad-Trans (approximately 41 versus 28%, respectively) (Fig. 4B). These data indicate that the viral IE86 protein in-


A.
HFF (p53+/+)
U2OS (p53+/+, p14ARF-/-)
Saos-2 (p53-/-)
Ad-IE86(10)
Ad-Trans(10)
Ad-IE86(10)
Ad-Trans(10)
Ad-IE86(10)
Ad-Trans(10)
Ad-p53(10)
a
b
c
d
DAPI
α-IE86
α-BrdU
Merge
α-p53
1
2
1
2
1
2
3


B.
Number of BrdU positive cells
100
90
80
70
60
50
40
30
20
10
0
HFF
U2OS
U373
Saos-2
Ad-IE86(10)
Ad-Trans(10)
Ad-p53(10)
C.
Number of BrdU positive cells
100
90
80
70
60
50
40
30
20
10
Parental
p53-/-
p21-/-
Ad-IE86(10)
Ad-Trans(10)
Ad-p53 or p21(10)

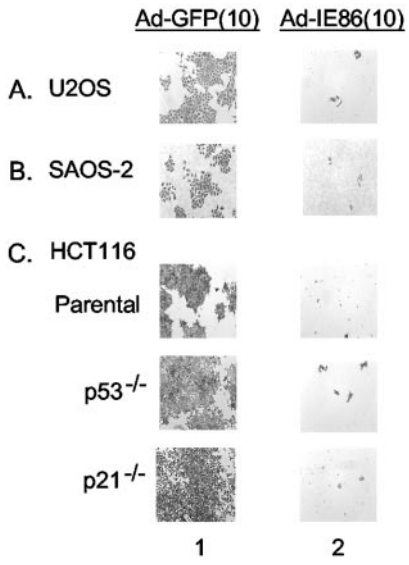


FIG. 3. Effect of the IE86 protein on cell division of $p53^{+/+}$, $p53^{-/-}$, or $p21^{-/-}$ cells. (A) U2OS cells, (B) Saos-2 cells, or (C) HCT116 cells and isogenic mutant cells were transduced at 10 PFU/cell with either Ad-GFP or Ad-IE86 in the presence of Ad-Trans (10 PFU/cell). Twenty thousand cells were replated in high-serum medium, incubated at 37°C for 6 days, fixed with formalin, and stained with methylene blue as described in Materials and Methods. A representative result of several experiments is shown.

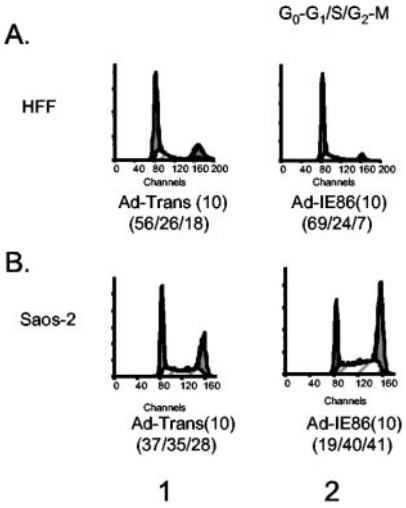


FIG. 4. Effect of IE86 protein on cell cycle progression in $p53^{+/+}$ or $p53^{-/-}$ cells. Serum-starved $p53^{+/+}$ HFF (A) or $p53^{-/-}$ Saos-2 (B) cells were transduced at 10 PFU/cell with either Ad-IE86 or Ad-Trans in the presence of Ad-Trans (10 PFU/cell). The cells were plated at low density and grown in high-serum medium for 48 h. After staining with propidium iodide, the DNA content of an equal number of cells was determined by fluorescence-activated cell sorter scanning. The numbers of cells in G_0-G_1/S/G_2-M are indicated.

hibits cell cycle progression at the G_2/M phase in p53 null Saos-2 cells.

Effect of IE86 protein on levels of cdk1 and cyclin B1. We tested the effect of the IE86 protein on cdk1/cyclin B1 activity. cdk1 and its regulatory subunit, cyclin B1, are required for cell cycle progression through the G_2/M phase (42, 57). Cyclin B1 is then degraded by a ubiquitin ligase called the anaphase-promoting complex (APC) or cyclosome, which inactivates cdk1/cyclin B1 and triggers an exit from mitosis to reenter the cell cycle (34, 37, 62, 63). HCMV has been reported to activate cdk1/cyclin B1 during the viral replication cycle (24, 47, 61). In addition, we reported that the viral IE86 protein increased cdk1 in HFF cells (54). We tested the levels of cdk1/cyclin B1 in Saos-2 cells. Serum-starved, p53 null Saos-2 cells were transduced with either Ad-IE86 or Ad-GFP and incubated with high-serum medium. After 0, 18, 24, and 48 h, cells were harvested and tested for the expression of cdk1 and cyclin B1 by Western blotting. The level of cdk1 protein increased at 18 h in Ad-IE86-transduced cells compared to that in Ad-GFP-transduced cells. At 48 h after transduction, cells transduced with Ad-IE86 had a twofold increase in the cdk1 protein level (Fig. 5A). To determine whether the increased level of cdk1 protein correlated with increased cdk1/cyclin B1 activity, an in vitro kinase assay was performed with cells prepared as described above. At 48 h, cells transduced with Ad-IE86 had threefold higher cdk1/cyclin B1 activity compared to cells transduced with Ad-GFP (Fig. 5B). The amount of cdk1 immunoprecipitated in cells transduced with Ad-IE86 was approximately equal to that in cells transduced with Ad-GFP (Fig. 5B). These data indicate that expression of the IE86 protein correlated with accumulation of the cells in the G_2/M phase and aberrantly high levels of cdk1/cyclin B1 activity.

DISCUSSION

The DNA tumor viruses interfere with the p53 pathway to avoid a cell cycle checkpoint system induced by deregulated E2F activity (11, 23, 38, 39, 49). HCMV is a DNA virus with both proliferative and antiproliferative effects on the cell. It induces quiescent HFF cells to reenter the cell cycle by activating E2F and cdks, but cellular DNA synthesis and cell division do not occur (7, 26, 56). Like the DNA tumor viruses, HCMV induces cell cycle progression by inhibiting the Rb family of proteins and activating the E2F transcription factors (7, 26, 56). The viral pp71, IE72, and IE86 proteins all induce cell cycle progression by inactivating the Rb family of proteins.

FIG. 2. Effect of the IE86 protein on cellular DNA synthesis in $p53^{+/+}$ or $p53^{-/-}$ cells. (A) Serum-starved cells were transduced at 10 PFU/cell with either Ad-IE86, Ad-Trans, or Ad-p53 in the presence of Ad-Trans (10 PFU/cell). Cells at low density were grown in high-serum medium with BrdU to allow reentry into the cell cycle. After 18 h, the cells were fixed and treated with antibody to either IE86, p53, or BrdU for immunofluorescence analysis as described in Materials and Methods. (B) The number of BrdU-positive cells in 100 cells as described above was determined with a microscope. (C) Serum-starved HCT116 cells and isogenic $p53^{-/-}$ or $p21^{-/-}$ mutant cells were transduced at 10 PFU/cell with either Ad-IE86, Ad-Trans, Ad-p53, or Ad-p21 in the presence of Ad-Trans (10 PFU/cell). Cells at low density were grown in high-serum medium with BrdU to allow reentry into the cell cycle. After 18 h, the cells were fixed and treated with antibody, and the mean number of BrdU-positive cells in 100 cells plus the standard error was determined. DAPI, 4′,6′-diamidino-2-phenylindole.

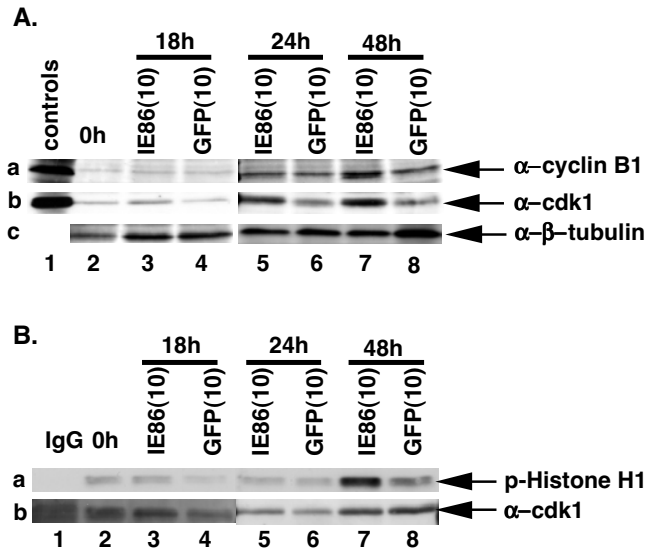


FIG. 5. Effect of IE86 protein on levels of cdk1 and cyclin B1 in $p53^{-/-}$ cells. (A) Serum-starved $p53^{-/-}$ Saos-2 cells were transduced at 10 PFU/cell with either Ad-IE86 or Ad-GFP in the presence of Ad-Trans (10 PFU/cell) and plated at a low density and grown in high-serum medium. After 0, 18, 24, and 48 h of incubation, whole-cell extract was harvested and subjected to Western blot analysis. Antibody to cyclin B1 (a), cdk1 (b), or β-tubulin (c) was used. The positive controls for cyclin B1 and cdk1 were 293 cells. (B) Equal amounts of cell extracts as described above were immunoprecipitated with anti-cdk1 antibody, assayed for in vitro kinase activity (a), and then subjected to Western blot analysis with anti-cdk1 antibody (b) as described in Materials and Methods.

The viral UL69 and IE86 proteins inhibit cellular DNA synthesis and arrest cells at the G_1/S interphase (7, 26, 56). HCMV inhibits cell cycle progression for at least two reasons. (i) HCMV utilizes cellular DNA precursors for its own DNA synthesis. (ii) HCMV prevents the S phase because during S phase viral IE gene expression is inhibited (15). The virus also activates the ATM pathway, which stabilizes p53 (48). While it is possible that other cellular proteins such as $p14^{ARF}$ have a role in inhibition of cellular DNA synthesis, our data indicate that inhibition of cellular DNA synthesis by the viral IE86 protein is dependent upon a functional p53 pathway.

In quiescent cells, the level of p21 is low but increases in response to mitogenic signals or p53 (41, 51). The viral IE86 protein increased the level of p21 by increasing the level of phospho-Ser_{15} p53. The IE86 protein induced phospho-Ser_{15} p53 and p21 in both HFF and U2OS cells, but not in AT cells. These data indicate that induction of the p53 pathway by the IE86 protein can be independent of $p14^{ARF}$ and dependent on ATM kinase, which phosphorylates p53. We propose that the IE86 protein induces the p53 pathway by deregulating E2F activity, activating ATM kinase, and stabilizing p53 by phosphorylation, which inhibits cellular DNA synthesis to favor HCMV replication.

In HFF cells, the p53 pathway induced by the IE86 protein is associated with cell cycle arrest, but not apoptosis. The prolonged cell cycle arrest by the IE86 protein causes the senescence phenotype in HFF cells (40).

Although cellular DNA synthesis is not inhibited by the IE86 protein in p53 mutant cells, the IE86 protein still inhibits cell division, but a mutated IE86 protein does not (36). To avoid any effect caused by mutant p53, we used Saos-2 cells, in which p53 is completely knocked out. In p53 null Saos-2 cells, the IE86 protein inhibited cell cycle progression in the G_2/M phase.

The HCMV IE86 protein up-regulates cdk1 (human cdc2) more than fourfold in quiescent HFF cells (54). It is possible that an increase in cdk1/cyclin B1 activity is the indirect effect of E2F activation by the IE86 protein because *cdk1* is an E2F-responsive gene. In Saos-2 cells, the expression of the IE86 protein correlates with arrest in the G_2/M phase and increased levels of cdk1/cyclin B1 activity. Since APC is required to degrade cyclin B1 to decrease cdk1 activity prior to reentering the cell cycle, the HCMV IE86 protein may interfere with APC to induce an aberrant level of cdk1 and cyclin B1.

Although HCMV inactivates the Rb pathway to induce cell cycle progression like the DNA tumor viruses, it does not allow $p53^{+/+}$ cells to enter the S phase. However, HCMV infection affects p53 because the cellular protein is sequestered in the nucleus in viral replication centers (17). Although the viral IE86 protein stabilizes p53 and activates p21, other proteins specified by the virus must inactivate p53 because HCMV infection causes a decrease in p21 levels (10, 40).

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